correct version of the affected text of the "Background of the Invention". The correct insertion is an exact copy of the original text which was previously filed in the United States Patent and Trademark Office in the aforementioned provisional application on December 23, 1999.

Moreover, it is clear upon closer reading that the present, uncorrected text is convoluted and incomplete. This flow is due to an inadvertent computer failure which apparently occurred when the text was reformatted to the PCT acceptable A4 DIN paper format, also filed in the U.S. version of the instant application.

Since the amended text does not add any new matter to the description of the claimed invention but merely clarifies the presentation of the background state of the art, the present amendment to repair an inadvertent deletion is deemed acceptable under the rules. (i.e. 37CFR 1.125(b)(1) and 1.121(1)(i) and (ii).

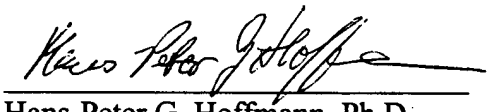
A marked-up copy of the Specification is appended hereto, substituting the pages 1 and 2 showing the matter being added to and the matter being deleted from the Specification page 2 of record (37 CFR 1.125(b)(2)), as well as marked pages 6, 7, and 11.

In order to account for the line shifts of the text per page which was caused by the present correction from page 1 through page 9, Applicant appends hereto an appropriately corrected unmarked copy of pages 1-9 of the instant Specification as replacement of the original pages 1-9. Unmarked page 11 has been appended to show the amended expression, -Tables D and E--. The Examiner is requested to insert the first nine pages in the Specification submitted in clear form without markings as to amended material, as replacement of the corresponding pending pages of record. Amended page 11 can also be used to replace the original page 11.

No fee is believed due with this submission. However, the Commissioner is authorized to charge any fee which may be due to Deposit Account No. 23-1703.

Dated: March 1, 2002

Respectfully submitted,



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Enclosure

1102865-0036

STABLE IMMUNOGENIC COMPOSITION FOR FROZEN STORAGE

This application claims priority from the provisional application Serial No. 60/173,022 which was filed on December 23, 1999.

Field of Invention

The invention is directed to a stable formulated immunogenic emulsion containing a combination of an antigen and an immunogenic carrier protein. More particularly, the invention is directed to a frozen emulsion which advantageously protects the immunogen during long-term storage.

Background of Invention

Immunization methodology has developed from the earlier methods of vaccination against invasive organisms or particles as an effective means for generating an immune defense to more recent approaches for regulating or controlling the physiological functions and reactions of the body. The immunogenic constructs can be administered in the form of an emulsion, also containing an oily vehicle and adjuvant for potentiation on the immune response as well as emulsifying and emulsion-stabilizing agents. The immunogenic emulsions are usually either the oil-in-water or water-in-oil variety.

Although water-in-oil emulsions have posed stability problems dependent on materials, salts, temperature and other factors, water-in-mineral oil emulsions have increasingly served as effective vehicles for vaccines. The best known emulsions of this type are known in the literature as the Freund's Adjuvants which have become effectively the emulsion standard. The Complete Freund's Adjuvant differs from the Incomplete Freund's Adjuvant in that the Complete Freund's Adjuvant comprises immune response potentiating tuberculin mycobacterium. However, since these mineral oil-based adjuvant forms are not well tolerated by the parentally immunized subject, different, more amenable, forms have been introduced especially for human use. For example, U.S. Patent No. 4,708,753 to Forsberg discloses a water-in-oil emulsion with a minor amount of emulsifying agent, wherein the oil phase is continuous. U.S. Patent No. 4,808,334 to Ezaki, et al. is directed to a process for compositions which are sterilized at high temperature and emulsified. U.S. Patent No. 4,960,814 to Wu et al. discloses a process to prepare a water-in-oil emulsion or, more particularly, a water-in-hydrophobic polymer emulsion. Injectable water-in-oil vaccine emulsions of low reactogenicity containing Montanide ISA 703 with 1.8% AMS are disclosed in co-assigned U.S. Patents No. 5,023,077, 5,468,494 and No. 5,688,506. U.S. Patents No. 5,422,109 and No. 5,424,067 to Brancq, et al. disclose an injectable vaccine emulsion comprising a metabolizable oil. WO 90/14837 discloses an adjuvant composition where the emulsion droplets are submicron size. EP 0187286 describes stable oily

adjuvant-emulsified vaccines composed of a paraffin oil, sorbitan monoleate and oxyethylene/oxypropylene polymer. U.S. Patent No. 5,376,369 to Allison, et al. discloses a vaccine adjuvant emulsion comprised of non-toxic polyols or olyl block polymer in the presence of a potentiating muramyldipeptide. U.S. Patent No. 5,679,355 to Alexander, et al. discloses vaccines containing non-ionic surfactant vesicles. U.S. Patent No. 5,109,026 to Hoskinson, et al. discloses vaccine formulations of water-in-oil emulsions containing polycationic polyelectrolyte immunoadjuvant and an oily substance, including, e.g., Drakeol, Markol, or any mixture of squalene and squalane. U.S. Patent No. 5,885,590 to Hunter et al. discloses injectable compositions of water-in-oil emulsions (and water-oil-water multiple emulsions) where the oily phase of the vaccine adjuvants can include squalene mostly together with a lesser amount of squalane. Under appropriate conditions immunization compositions can be enhanced by combining them with the immunological adjuvant consisting of a saline suspension of lyzed filamentous Amycolate bacteria cells.

Emulsions are formed in several different ways, such as, e.g., by mechanical action or spontaneously. Stabilization of water-in-oil emulsions formulated with a hormone peptide immunogen should preferably be achieved without applying heat, x-ray, cross-linking agents, irritating or toxic solvents and oils, in order to be pharmaceutically acceptable. Emulsion formulations of immunogens such as, e.g., anti-peptide hormone, are effective components of vaccination success. Anti-peptide hormone vaccines are herein defined as conjugates of an immunogenic carrier protein to a peptide hormone antigen comprising a hormone-immunomimic peptide.

An important practical consideration for applications of the anti-hormone vaccine technology is the shelf-life of the water-in-oil emulsion-based immunogenic composition after its manufacture and before its end use. The present refrigerated shelf-life of such formulated emulsions is about 3-6 months at about 4°C. In view of the expense of the immunogen and need for the immunogenic composition to be available for extended periods of time of treatment, it has been found desirable to obtain long term stable storage capability. The major limiting factor of a prolonged storage of the formulated emulsion vaccine has been the elution of immunomimic peptide from the immunogenic carrier.

It has now been discovered that there are several adjuvant oily substances useful as vehicles for emulsions which have been stable when frozen stored for a considerable time.

SUMMARY OF THE INVENTION

The present invention provides an emulsified immunogenic composition which has the advantageous capability of long-term frozen storage.

According to an embodiment of the invention, it has been discovered that certain emulsified immunogenic compositions provide long-term frozen storage stability. It has been further discovered that the frozen storage of the emulsion according to the invention may be extended for more than the usual time, such as about one half year, to about one year or more.

The frozen storage capability of the inventive emulsion composition comprises metabolizable oily substances of vehicles which are pharmaceutically acceptable. The inventive emulsion can be formulated with an oily substance or vehicles containing a mixture of squalene and squalane. More particularly, an oily substance according to the present invention for producing an immunogenic emulsion which is stable during frozen storage over a wide range of freezing temperature, is selected from Montanide ISA 25, Montanide ISA 703, Montanide ISA 719, or Montanide ISA 720.

Specifically, the emulsion compositions according to this invention are found stable at the temperatures -18°, -23° and -70°C. Furthermore, the inventive composition can provide stable storage capability for an immunogen which may comprise epitopes of non-peptide or peptide antigenic moietes.

One of the embodiments of the present invention comprises a stable water-in-oil emulsion comprising a peptide hormone or peptide fragment thereof which is conjugated to an immunogenic carrier protein. Another embodiment of the invention comprises stable oil-in-water emulsion.

The conjugate in the inventive water-in-oil emulsion may comprise a synthetic hormone-immunomimic peptide linked to an immunogenic carrier.

A use of the composition includes parenteral administration. For example, in accordance with the invention, an injectable immunogen emulsion is formulated for immunization of an animal or human against its own hormone epitopes, comprising an emulsion with an aqueous phase comprising an antigen having low or negligible immunogenicity which is conjugated to an immunogenic protein carrier and an oily vehicle comprising a metabolizable oily substance or a mixture of different suitable oily substances.

Furthermore, according to the invention, the emulsion mixture remains stable after several cycles of freezing and thawing. The inventive emulsion containing the suitable oily substances have been found to be stable after undergoing several freeze/thaw cycles.

In particular, the pharmaceutically acceptable oil vehicle comprises a mixture of metabolizable squalene and squalane, and surfactant additives, such as emulsifiers and emulsion stabilizers. Furthermore, the squalene and/or squalane mixture can comprise one or more vehicles selected from the group consisting of Montanide ISA 25, Montanide ISA 703,

Montanide ISA 719, and Montanide ISA 720. According to embodiment, a surfactant emulsifier can be Mannide monooleate and a surfactant emulsion stabilizer can be polyoxy-40-hydrogenated castor oil.

An embodiment of the invention provides a stable emulsion suitable for frozen storage containing a gastrin peptide or fragment thereof conjugated to an immunogenic carrier. Another embodiment provides a stable emulsion suitable for frozen storage containing a GnRH epitope or part thereof conjugated to an immunogenic carrier.

An inventive embodiment can provide a stable emulsion suitable for frozen storage containing a gastrin 17 epitope or a gastrin 34 epitope, which is conjugated to an immunogenic carrier, such as, e.g., diphtheria toxoid, tetanus toxoid, bovine serum albumin, or keyhole limpet hemocyanin, horseshoe crab hemocyanin, ovalbumin, dextran, or immunogenic fragments thereof.

Another preferred embodiment provides a stable emulsion suitable for frozen storage containing a synthetic gonadotropin releasing hormone (GnRH) peptide or fragment thereof, which is conjugated to an immunogenic carrier, such as e.g., diphtheria toxoid, tetanus toxoid, bovine serum albumin, keyhole limpet hemocyanin, horseshoe crab hemocyanin, ovalbumin or immunogenic fragments thereof.

Moreover, the frozen emulsion of this invention would remain stable for a storage period ranging up to at least 12 months at freezing temperatures ranging from about -18°C to about -80°C. The preferred frozen emulsions of this invention remain stable for a storage period of at least 12 months at temperatures of about -18°C, -23°C or -70°C.

One of the embodiments of the invention comprises a stable emulsion suitable for frozen storage comprising Montanide ISA 703, Montanide ISA 719 or Montanide ISA 720, which comprises pharmaceutically acceptable components, as described below. For example, the formulated emulsion may contain Montanide ISA 703, Montanide ISA 719 or Montanide ISA 720 and a synthetic G17 peptide-spacer analogue conjugated to an immunogenic moiety.

In particular, an emulsion can contain Montanide ISA 703 and human G17(1-9)-DT conjugate. Analigunot of the emulsion may contain about 0.5 mg/ml of conjugate.

Furthermore, it has been found that the immunogenic emulsion of the invention remains active when stored for an extended period at a temperature ranging from about -18° C to about -80°C, even after several freeze/thaw cycles in succession. For example, the emulsion globules can remain at about 97% of droplet size of less than 1μm diameter after five freeze/thaw cycles from -18° C. Furthermore, the emulsion of this embodiment comprises an intact conjugate immunogen content of about 97.5% after five -18° C freeze/thaw cycles or about 97.5% after five -70° C freeze/thaw cycles.

In addition, the formulated stable emulsion globules of the embodiment have retained at least 97% of their original size during frozen storage at least for 12 months.

It has been found that the anti-gastrin immunogenic emulsion of the invention surprisingly shows an improved anti-gastrin immunogenicity after one freezing/thawing cycle at -18° C. Thus, the improved immunogenicity of the inventive emulsion will significantly increase the antibody titer as compared to the starting material.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the results of percent purity of hG17 (9)-DT conjugate in the aqueous phase extract from the emulsion after storage at -70°, -18°, 4° and 25°C, analyzed by exclusion chromatography with a TSK-GEL G3000SW$_{XL}$ Column;

Fig. 2 illustrates the results of the material of Fig. 1, by exclusion chromatography with a TSK-GEL G2000SW column;

Fig. 3 illustrates percent conjugate release rate of the emulsion stored for up to 12 months at 4°C;

Fig. 4 illustrates the conjugate release rate at 25°C;

Fig. 5 illustrates the conjugate release rate at -70°C;

Fig. 6 illustrates the conjugate release rate at -18°C;

Fig. 7 illustrates the immunogenicity of emulsion after storage at 4°C for zero, 3, 6 and 12 months;

Fig. 8 illustrates the immunogenicity of emulsion after storage at 25°C for zero, 3, 6 and 12 months;

Fig. 9 illustrates the immunogenicity of emulsion after storage at -70°C for zero, 3, 6 and 12 months;

Fig. 10 illustrates the immunogenicity of emulsion after storage at -18°C for zero, 3, 6 and 12 months;

Fig. 11 illustrates the local tolerance or reactogenicity of emulsion stored at 4°C for zero, 3, 6 and 12 months;

Fig. 12 illustrates the local tolerance or reactogenicity of emulsion stored at 25°C for zero, 3, 6 and 12 months;

Fig. 13 illustrates the local tolerance or reactogenicity of emulsion stored at -70°C for zero, 3, 6 and 12 months; and

Fig. 14 illustrates the local tolerance or reactogenicity of emulsion stored at -18°C for zero, 3, 6 and 12 months.

DETAILED DESCRIPTION OF THE INVENTION

According to this invention, immunizations against non-peptide and peptide antigens have utilized emulsions of an aqueous phase containing an immunomimic epitope conjugated to a pharmaceutically acceptable immunogenic carrier and a lipid phase containing a pharmaceutically acceptable oily substance, wherein the emulsions are formulated so as to be stable during storage with repeated freezing/thawing cycles. Pharmaceutically acceptable oily vehicles are metabolizable and understood to be well tolerated systemically by the human, as well as less irritating at the injection site of the human by showing low reactogenicity.

In accordance with the experiments described below the emulsions comprise oil-in-water, water-in-oil, and water-in-oil-in-water configurations.

Immunogenic emulsions have been disclosed in e.g., U.S. Patent Nos. 5,422,109, 5,424,067, 5,885,590, 5,109,026, 4,708,753, 4,808,334, and 4,960,814, which are incorporated herein in their entirety by reference. More specifically, immunizations with Gastrin or GnRH immunogens in the form of injectable water-in-oil emulsions have been described in co-assigned U.S. Patent No. 5,468,494, 5,023,077, 5,609,870 and 5,688,506, which are herewith incorporated in this application by reference in their entirety.

Although freezing the emulsion was originally employed as a gentle method to separate the conjugate-bearing aqueous phase from the emulsion for easier sampling and analysis, the emulsions preparations according to this invention surprisingly did not break down even when expired to several freeze-thaw cycles. This stability under the repeated freeze/thaw stress was all the more surprising because frozen storage of emulsions had not been previously considered an option. Freezing and thawing was generally held to be detrimental to the stability of emulsions, perhaps leading to disruption of conjugates and aggregation or separation of emulsion components. Moreover, when it was also found that solutions of conjugates in PBS (phosphate buffered saline) could be frozen with little loss of integrity of the conjugate of an immunogenic carrier coupled peptide, experiments were conducted to determine if it was also possible to stably store the frozen formulated emulsion. For example, the anti-gastrin formulated emulsion was tested by storage at about -70° C (as provided by a deep freezer) or about -18° C (as provided by a general freezer temperature). Accordingly, the emulsions of this invention have been formulated so as to keep the vaccine intact in long-term frozen storage.

In the context of the anti-hormone immunogenic embodiment of this invention, the conjugated immunogens can be synthetic peptides or fragments thereof, which may also be extended with spacer peptides, covalently attached to immunogenic protein carriers. The immunogenic carrier can be diphtheria toxoid, tetanus toxoid, a solvent extract of filamentous Amycolate or H. Pertussis, keyhole limpet hemocyanin, horseshoe crab hemocyanin, bovine serum albumin, ovalbumin, or dextran or immunogenic fragments thereof.

Dextran is a purified polysaccharide product of Leuconostoc mesenteroides strain B-512. The preferred oligosaccharide molecular weights of 64,000-76,000 are used as conjugate carrier. Other immunization enhancing additives include aluminum phosphate serving as adsorbents for DT or TT.

The peptide or the fragment of the peptide is selected to comprise an immunomimic region of the target hormone epitope. The immunogenic conjugates are administered in the form of injectable water-in-oil or oil-in-water emulsions.

Comparative tests described below have demonstrated that certain metabolizable Montanide ISA preparations (Seppic, France) has been stable during frozen storage at -23°C or -70°C. The select group of Montanide ISA preparations include Montanide ISA 25, Montanide ISA 703, Montanide ISA 719 and Montanide ISA 720. In particular, pharmaceutically acceptable Montanide ISA 703 has been found an especially useful oily vehicle for forming a stable emulsion that is effective for immunogenic compositions. Alternatively, other metabolizable combinations of squalene/squalane and additives can be utilized which are less irritating or more gentle, and thus more amenable to the human.

A composition according to this invention comprising 0.5 mg/ml of the above described immunogenic conjugates in Montanide ISA 703 has been found to form a stable emulsion which is suitable for storage at temperatures below the freezing point. In fact, as described below, the formulated vaccine emulsion was found to remain stable when frozen for several months, up to at least about one year. Thawed-out emulsions maintained visual integrity. Storage of immunogenic emulsions at different temperatures and after one or more freeze-thaw cycles under the storage conditions described below, did not significantly affect the conjugate integrity or cause oil phase separation in the emulsion. In fact, the emulsion globules did not show any significant aggregation, did not undergo a significant shift in a size distribution, or a significant loss of desirable uniformity of conformation by exceeding the preferred initial 1μm size.

In addition, the immunogenicity of the emulsion was significantly increased after at least one frozen storage cycle at -18° C. More specifically, immunization with the frozen sample

stored at -18° C was found to generate antibody titers which are about twice that of the emulsion which was not frozen.

Immunization emulsions suitable for frozen storage can be used with any of the anti-gastrin or anti-GnRH immunogenic conjugates, disclosed in U.S. Patent No. 5,023,077 and 5,688,506, respectively.

The following examples illustrate the analysis of the inventive emulsions on the basis of certain criteria for their stability. Examples 1 and 2 employed the same preparations of emulsion.

The analysis included several categories such as appearance, particle size of the emulsion globules, conjugate immunogen purity in the extracted aqueous phase, release rate of conjugate from emulsion *in vitro,* as well as immunogenicity and injection site tolerance *in vivo.*

Example 1 - Freeze-Thaw Cycles

1. Preparation of Emulsions

The following procedure for forming an immunogenic emulsion is described in the co-assigned U.S. Patent No. 5,023,077. In particular, the immunogenic hormone peptide conjugate (i.e., gastrin peptide immunogen conjugate) was dissolved in phosphate buffered saline at pH 7.2 ("PBS") to produce the initial aqueous phase. The initial aqueous phase of the conjugate was dissolved in PBS at a concentration of 1.882 mg/ml. The sterile emulsion was prepared by combining the aqueous phase containing the conjugate with sterile nontoxic or non-irritant oily vehicle phase, such as, e.g., Montanide ISA 703, at a ratio of 70:30 oil to aqueous phase (w/w) to comprise the final immunogenic emulsion concentration of 0.5 mg/ml. In accordance with the present protocol, emulsions were prepared by mixing 410 ml in the Silverson 500 ml mixing head, at 8,000 rpm for 4 minutes using Montanide ISA 703 as vehicle, the conjugate was hG17(1-9)Ser9-DT.

2. Freeze-Thaw Treatments

The vials (10 per temperature tested) were stored at -70°C (Ultra-Low Freezer), and -18°C (standard freezer).

The samples were assessed for their appearance (Tables A and B), globule size (Table C), and conjugate concentration and purity. The vials with frozen emulsions were removed from the respective freezers and allowed to come to room temperature. The vials were mixed by moderate shaking. One vial from each temperature was kept at 4 °C for testing, while the others were used to repeat the freeze/thaw procedure at the respective temperatures. The vials were subjected to 0-5 freeze-thaw cycles.

3. Appearance

The appearance of the emulsion was noted immediately after samples were removed from either -70° or -18°C, and again after thawing to room temperature and mixed by shaking. When stored at -70°C, all components of the emulsion appeared frozen. No difference in appearance was found between the frozen and subsequently thawed emulsions and the pre-freezing emulsion control. Re-suspension by shaking was not required to maintain the original appearance.

However, not all components of the emulsion were frozen when stored at -18°C. There was a noticeable difference between the frozen and subsequently thawed emulsions in appearance from the emulsion prior to freezing. But only moderate shaking was required for uniform re-suspension of the emulsion.

Following a specific number of freeze/thaw cycles (as indicated), the samples were stored at 4°C. Under these conditions, the samples maintained a white semi-viscous appearance with no signs of settling or separation.

4. Globular Size Distribution (Table C)

Globule size determination was performed on all samples from both freezing temperatures and the cold storage non-frozen control (4°C). There was no change in globule size distribution after one freeze/thaw cycle, although, there was a slight increase in the percentage of globule size greater than 1 μm, ranging up to 2.5% after 5 freeze/thaw cycles.

Table C. Globule Size Distribution Results

Sample	Percent ≥ 1 μm
Control Emulsion	0.40 %
-18°C one F/T cycle	0.45 %
-18°C two F/T cycles	1.85 %
-18°C three F/T cycles	0.89 %
-18°C four F/T cycles	2.45 %
-18°C five F/T cycles	2.50 %
-70°C one F/T cycle	0.35 %
-70°C two F/T cycles	2.17 %
-70°C three F/T cycles	2.14 %
-70°C four F/T cycles	2.16 %
-70°C five F/T cycles	2.5 %

5. HPLC Analysis

To analyze the conjugate in the emulsions by HPLC, the conjugate-bearing aqueous phase was first extracted from the emulsion by treatment of an aliquot of emulsion with an equal volume of isobutanol. Following centrifugation (4,000 x g for 10 min.) to separate the aqueous and oil phases, the aqueous phase was collected and tested by HPLC. The HPLC conditions were: flow rate = 0.5 ml/min.; butter = PBS, pH = 7.2; run duration = 35 min.; sample volume = 0.010 ml; column = TSK-GEL® G3000 SW_{xl} (10 mm x 300 mm); room temperature; injection volume =sample volume. The integrated data from the analyses was used to calculate the purity (% intact) of the conjugate extracted from the emulsions.

A retained aliquot of the aqueous phase (used to prepare the anti-gastrin immunogen) was used as an aqueous control for concentration determination (Stock conjugate lot no. G1297-5). Comparison of the chromatograms for samples subjected to five freeze/thaw cycles with chromatograms for the control showed that freezing had no effect upon the elution profile of conjugate in the sample. Moreover, under both storage conditions, there were no changes in conjugate concentration or purity after 5 freeze/thaw cycles, as seen in Tables D and E.

Patents No. 5,023,077, 5,468,494 and No. 5,688,506. U.S. Patents No. 5,422,109 and No. 5,424,067 to Brancq, et al. disclose an injectable vaccine emulsion comprising emulsifying agent, wherein the oil phase is continuous. U.S. Patent No. 4,808,334 to Ezaki, et al. is directed to a process for compositions which are sterilized at high temperature and emulsified. U.S. Patent No. 4,960,814 to Wan et al. discloses a process to prepare a water-in-oil emulsion or, more particularly, a water-in-hydrophobic polymer emulsion. Injectable water-in-oil vaccine emulsions of low reactogenicity containing Montanide ISA 703 with 1.8% AMS are disclosed in co-assigned U.S. Patents No. 5,023,077, 5,468,494 and No. 5,688,506. U.S. Patents No. 5,422,109 and No. 5,424,067 to Brancq, et al. disclose an injectable vaccine emulsion comprising including, e.g., Drakeol, Markol, or any mixture of squalene and squalane. U.S. Patent No. 5,885,590 to Hunter et al. discloses injectable compositions of water-in-oil emulsions (and water-oil-water multiple emulsions) where the oily phase of the vaccine adjuvants can include squalene mostly together with a lesser amount of squalane. Under appropriate conditions immunization compositions can be enhanced by combining them with the immunological adjuvant consisting of a saline suspension of lyzed filamentous Amycolate bacteria cells.

Emulsions are formed in several different ways, such as, e.g., by mechanical action or spontaneously. Stabilization of water-in-oil emulsions formulated with a hormone peptide immunogen should preferably be achieved without applying heat, x-ray, cross-linking agents, irritating or toxic solvents and oils, in order to be pharmaceutically acceptable. Emulsion formulations of immunogens such as, e.g., anti-peptide hormone, are effective components of vaccination success. Anti-peptide hormone vaccines are herein defined as conjugates of an immunogenic carrier protein to a peptide hormone antigen comprising a hormone-immunomimic peptide.

An important practical consideration for applications of the anti-hormone vaccine technology is the shelf-life of the water-in-oil emulsion-based immunogenic composition after its manufacture and before its end use. The present refrigerated shelf-life of such formulated emulsions is about 3-6 months at about 4°C. In view of the expense of the immunogen and need for the immunogenic composition to be available for extended periods of time of treatment, it has been found desirable to obtain long term stable storage capability. The major limiting factor of a prolonged storage of the formulated emulsion vaccine has been the elution of immunomimic peptide from the immunogenic carrier.

It has now been discovered that there are several adjuvant oily substances useful as vehicles for emulsions which have been stable when frozen stored for a considerable time.

Fig. 14 illustrates the local tolerance or reactogenicity of emulsion stored at -18°C for zero, 3, 6 and 12 months.

DETAILED DESCRIPTION OF THE INVENTION

According to this invention, immunizations against non-peptide and peptide antigens have utilized emulsions of an aqueous phase containing an immunomimic epitope conjugated to a pharmaceutically acceptable immunogenic carrier and a lipid phase containing a pharmaceutically acceptable oily substance, wherein the emulsions are formulated so as to be stable during storage with repeated freezing/thawing cycles. Pharmaceutically acceptable oily vehicles are metabolizable and understood to be well tolerated systemically by the human, as well as less irritating at the injection site of the human by showing low reactogenicity.

In accordance with the experiments described below the emulsions comprise oil-in-water, water-in-oil, and water-in-oil-in-water configurations.

Immunogenic emulsions have been disclosed in e.g., U.S. Patent Nos. 5,422,109, 5,424,067, 5,885,590, 5,109,026, 4,708,753, 4,808,334, and 4,960,814, which are incorporated herein in their entirety by reference. More specifically, immunizations with Gastrin or GnRH immunogens in the form of injectable water-in-oil emulsions have been described in co-assigned U.S. Patent No. 5,468,494, 5,023,077, 5,609,870 and 5,688,506, which are herewith incorporated in this application by reference in their entirety.

Although freezing the emulsion was originally employed as a gentle method to separate the conjugate-bearing aqueous phase from the emulsion for easier sampling and analysis, the emulsions preparations according to this invention surprisingly did not break down even when expired to several freeze-thaw cycles. This stability under the repeated freeze/thaw stress was all the more surprising because frozen storage of emulsions had not been previously considered an option. Freezing and thawing was generally held to be detrimental to the stability of emulsions, perhaps leading to disruption of conjugates and aggregation or separation of emulsion components. Moreover, when it was also found that solutions of conjugates in PBS (phosphate buffered saline) could be frozen with little loss of integrity of the conjugate of an immunogenic carrier coupled peptide, experiments were conducted to determine if it was also possible to stably store the frozen formulated emulsion. For example, the anti-gastrin formulated emulsion was tested by storage at about -70° C (as provided by a deep freezer) or about -18° C (as provided by a general freezer temperature). Accordingly, the emulsions of this invention have been formulated so as to keep the vaccine intact in long-term frozen storage.

In the context of the anti-hormone immunogenic embodiment of this invention, the conjugated immunogens can be synthetic peptides or fragments thereof, which may also be extended

with spacer peptides, covalently attached to immunogenic protein carriers. The immunogenic carrier can be diphtheria toxoid, tetanus toxoid, a solvent extract of filamentous Amycolate or H. Pertussis, keyhole limpet hemocyanin, horseshoe crab hemocyanin, bovine serum albumin, ovalbumin, or dextran or immunogenic fragments thereof.

Dextran is a purified polysaccharide product of Leuconostoc mesenteroides strain B-512. The preferred oligosaccharide molecular weights of 64,000-76,000 are used as conjugate carrier. Other immunization enhancing additives include aluminum phosphate which serve as adsorbents for DT or TT.

The peptide or the fragment of the peptide is selected to comprise an immunomimic region of the target hormone epitope. The immunogenic conjugates are administered in the form of injectable water-in-oil or oil-in-water emulsions.Comparative tests described below have demonstrated that certain metabolizable Montanide ISA preparations (Seppic, France) has been stable during frozen storage at -23°C or -70°C. The select group of Montanide ISA preparations include Montanide ISA 25, Montanide ISA 703, Montanide ISA 719 and Montanide ISA 720. In particular, pharmaceutically acceptable Montanide ISA 703 has been found an especially useful oily vehicle for forming a stable emulsion that is effective for immunogenic compositions. Alternatively, other metabolizable combinations of squalene/squalane and additives can be utilized which are less irritating or more gentle, and thus more amenable to the human.

A composition according to this invention comprising 0.5 mg/ml of the above described immunogenic conjugates in Montanide ISA 703 has been found to form a stable emulsion which is suitable for storage at temperatures below the freezing point. In fact, as described below, the formulated vaccine emulsion was found to remain stable when frozen for several months, up to at least about one year. Thawed-out emulsions maintained visual integrity. Storage of immunogenic emulsions at different temperatures and after one or more freeze-thaw cycles under the storage conditions described below, did not significantly affect the conjugate integrity or cause oil phase separation in the emulsion. In fact, the emulsion globules did not show any significant aggregation, did not undergo a significant shift in a size distribution, or a significant loss of desirable uniformity of conformation by exceeding the preferred initial 1μm size.

In addition, the immunogenicity of the emulsion was significantly increased after at least one frozen storage cycle at -18° C. More specifically, immunization with the frozen sample stored at -18° C was found to generate antibody titers which are about twice that of the emulsion which was not frozen.

Immunization emulsions suitable for frozen storage can be used with any of the anti-gastrin or anti-GnRH immunogenic conjugates, disclosed in U.S. Patent No. 5,023,077 and 5,688,506,

1102865-0036

A STABLE IMMUNOGENIC COMPOSITION FOR FROZEN STORAGE

This application claims priority from the provisional application Serial No. 60/173,022 which was filed on December 23, 1999.

Field of Invention

The invention is directed to a stable formulated immunogenic emulsion containing a combination of an antigen and an immunogenic carrier protein. More particularly, the invention is directed to a frozen emulsion which advantageously protects the immunogen during long-term storage.

Background of Invention

Immunization methodology has developed from the earlier methods of vaccination against invasive organisms or particles as an effective means for generating an immune defense to more recent approaches for regulating or controlling the physiological functions and reactions of the body. The immunogenic constructs can be administered in the form of an emulsion, also containing an oily vehicle and adjuvant for potentiation on the immune response as well as emulsifying and emulsion-stabilizing agents. The immunogenic emulsions are usually either the oil-in-water or water-in-oil variety.

Although water-in-oil emulsions have posed stability problems dependent on materials, salts, temperature and other factors, water-in-mineral oil emulsions have increasingly served as effective vehicles for vaccines. The best known emulsions of this type are known in the literature as the Freund's Adjuvants which have become effectively the emulsion standard. The Complete Freund's Adjuvant differs from the Incomplete Freund's Adjuvant in that the Complete Freund's Adjuvant comprises immune response potentiating tuberculin mycobacterium. However, since these mineral oil-based adjuvant forms are not well tolerated by the parentally immunized subject, different, more amenable, forms have been introduced especially for human use. For example, U.S. Patent No. 4,708,753 to Forsberg discloses a water-in-oil emulsion with a minor amount of emulsifying agent, wherein the oil phase is continuous. U.S. Patent No. 4,808,334 to Ezaki, et al. is directed to a process for compositions which are sterilized at high temperature and emulsified. U.S. Patent No. 4,960,814 to Wu et al. discloses a process to prepare a water-in-oil emulsion or, more particularly, a water-in-hydrophobic polymer emulsion. Injectable water-in-oil vaccine emulsions of low reactogenicity containing Montanide ISA 703 with 1.8% AMS are disclosed in co-assigned U.S. Patents No. 5,023,077, 5,468,494 and No. 5,688,506. U.S. Patents No. 5,422,109 and No. 5,424,067 to Brancq, et al. disclose an injectable vaccine emulsion comprising a metabolizable oil. WO 90/14837 discloses an adjuvant composition where the emulsion droplets are submicron size. EP 0187286 describes stable oily

adjuvant-emulsified vaccines composed of a paraffin oil, sorbitan monoleate and oxyethylene/oxypropylene polymer. U.S. Patent No. 5,376,369 to Allison, et al. discloses a vaccine adjuvant emulsion comprised of non-toxic polyols or olyl block polymer in the presence of a potentiating muramyldipeptide. U.S. Patent No. 5,679,355 to Alexander, et al. discloses vaccines containing non-ionic surfactant vesicles. U.S. Patent No. 5,109,026 to Hoskinson, et al. discloses vaccine formulations of water-in-oil emulsions containing polycationic polyelectrolyte immunoadjuvant and an oily substance, including, e.g., Drakeol, Markol, or any mixture of squalene and squalane. U.S. Patent No. 5,885,590 to Hunter et al. discloses injectable compositions of water-in-oil emulsions (and water-oil-water multiple emulsions) where the oily phase of the vaccine adjuvants can include squalene mostly together with a lesser amount of squalane. Under appropriate conditions immunization compositions can be enhanced by combining them with the immunological adjuvant consisting of a saline suspension of lyzed filamentous Amycolate bacteria cells.

Emulsions are formed in several different ways, such as, e.g., by mechanical action or spontaneously. Stabilization of water-in-oil emulsions formulated with a hormone peptide immunogen should preferably be achieved without applying heat, x-ray, cross-linking agents, irritating or toxic solvents and oils, in order to be pharmaceutically acceptable. Emulsion formulations of immunogens such as, e.g., anti-peptide hormone, are effective components of vaccination success. Anti-peptide hormone vaccines are herein defined as conjugates of an immunogenic carrier protein to a peptide hormone antigen comprising a hormone-immunomimic peptide.

An important practical consideration for applications of the anti-hormone vaccine technology is the shelf-life of the water-in-oil emulsion-based immunogenic composition after its manufacture and before its end use. The present refrigerated shelf-life of such formulated emulsions is about 3-6 months at about 4°C. In view of the expense of the immunogen and need for the immunogenic composition to be available for extended periods of time of treatment, it has been found desirable to obtain long term stable storage capability. The major limiting factor of a prolonged storage of the formulated emulsion vaccine has been the elution of immunomimic peptide from the immunogenic carrier.

It has now been discovered that there are several adjuvant oily substances useful as vehicles for emulsions which have been stable when frozen stored for a considerable time.

SUMMARY OF THE INVENTION

The present invention provides an emulsified immunogenic composition which has the advantageous capability of long-term frozen storage.

4. Globular Size Distribution (Table C)

Globule size determination was performed on all samples from both freezing temperatures and the cold storage non-frozen control (4°C). There was no change in globule size distribution after one freeze/thaw cycle, although, there was a slight increase in the percentage of globule size greater than 1μm, ranging up to 2.5% after 5 freeze/thaw cycles.

Table C. Globule Size Distribution Results

Sample	Percent ≥ 1 μm
Control Emulsion	0.40 %
-18°C one F/T cycle	0.45 %
-18°C two F/T cycles	1.85 %
-18°C three F/T cycles	0.89 %
-18°C four F/T cycles	2.45 %
-18°C five F/T cycles	2.50 %
-70°C one F/T cycle	0.35 %
-70°C two F/T cycles	2.17 %
-70°C three F/T cycles	2.14 %
-70°C four F/T cycles	2.16 %
-70°C five F/T cycles	2.5 %

5. HPLC Analysis

To analyze the conjugate in the emulsions by HPLC, the conjugate-bearing aqueous phase was first extracted from the emulsion by treatment of an aliquot of emulsion with an equal volume of isobutanol. Following centrifugation (4,000 x g for 10 min.) to separate the aqueous and oil phases, the aqueous phase was collected and tested by HPLC. The HPLC conditions were: flow rate = 0.5 ml/min.; butter = PBS, pH = 7.2; run duration = 35 min.; sample volume = 0.010ml; column = TSK-GEL® G3000 SW_{xl} (10 mm x 300 mm); room temperature; injection volume =sample volume. The integrated data from the analyses was used to calculate the purity (% intact) of the conjugate extracted from the emulsions.

A retained aliquot of the aqueous phase (used to prepare the anti-gastrin immunogen) was used as an aqueous control for concentration determination (Stock conjugate lot no. G1297-5). Comparison of the chromatograms for samples subjected to five freeze/thaw cycles with chromatograms for the control showed that freezing had no effect upon the elution profile of conjugate in the sample. Moreover, under both storage conditions, there were no changes in conjugate concentration or purity after 5 freeze/thaw cycles, as seen in [Tables 4 and 5] Tables D and E.